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Summary:

This study investigates the non-specific immunological effects of DTP and measles vaccination of healthy African infants. The results indicate sex-differential effects on both innate and adaptive immunity, providing the first mechanistic insights into how these vaccines alter infectious disease susceptibility.

ABSTRACT

Vaccines can have non-targeted heterologous effects which manifest as increased protection against non-vaccine infections, as described for measles vaccine (MV); or increased susceptibility to infections and death, as described following diphtheria-tetanus-whole cell pertussis (DTP) vaccination. The mechanisms are unknown and high quality immunological studies are lacking. This study was designed to investigate the heterologous effects of MV and DTP in 302 Gambian infants. The results support an immunosuppressive effect of DTP on innate pro-inflammatory responses and T cell immunity in females. Males but not females receiving MV had enhanced pro-inflammatory innate responses. The results point to modified signalling via Toll-like receptor (TLR4) as a possible mechanism for the effects on innate immunity. When both vaccines were administered together, PPD responses were enhanced in females but down-regulated in males. Collectively these data indicate immunological effects that could account for heterologous effects of MV and DTP, to take forward into prospective trials.

INTRODUCTION

Background

Multiple epidemiological studies have described heterologous effects of vaccines on infectious disease susceptibility and all-cause mortality [1-4]. Bacille Calmette-Guérin (BCG) vaccinated low-birth-weight neonates had a 45% lower all-cause mortality compared to unvaccinated controls in a randomized trial conducted in Guinea Bissau [5]; and an additional early measles vaccine (MV) at 4.5 months was associated with reduced mortality in the absence of measles infections in a randomized trial in Guinea-Bissau [6]. A recent population-level data analysis suggested that MV protects against all-cause mortality by preventing prolonged immunosuppression caused by wild type measles infection [7]. However, if true, then censoring for measles infection should prevent beneficial effects of MV, which is not the case in numerous epidemiological studies [8].

A review of all available studies where mortality was assessed in the context of diphtheria-tetanus-whole cell pertussis (DTP) vaccination suggests that DTP is associated with increased mortality, particularly in females [9]. Several observational studies suggest that receiving DTP with MV is associated with higher mortality compared to receiving MV only, with males generally more susceptible [10, 11].

A systematic review for non-specific effects of vaccines commissioned by WHO concluded that the epidemiological evidence supports beneficial heterologous effects of MV; but found insufficient evidence to confirm or refute deleterious heterologous effects of DTP [12], although the analytical principles used have been questioned

[13]. The need to explore and understand the immunological mechanisms was emphasized in the WHO review.

We used state-of-the-art methodology to elucidate the immunological mechanisms that might account for beneficial effects of MV and deleterious effects of DTP in a prospective randomized trial, and further analysed the effect of administering both vaccines simultaneously.

METHODS

Study Subjects

Infants attending for routine vaccination were recruited at four months of age at Sukuta Health Centre, a peri-urban area 20 km from the coast of The Gambia. Eligibility criteria included being healthy, apyrexial (<37.5°C), normal weight-for-age, and all recommended vaccines received to date. The study was approved by the Joint Gambia Government/MRC Ethics Committee (project number SCC1085). Written informed consent was provided by the parent/guardian of the child.

Study Design

Four month old infants were block randomized to one of three vaccine groups in a prospective unblinded study. At 4 months of age group 1 received their third dose of diphtheria-tetanus-whole cell pertussis (DTP), hepatitis B vaccine (HBV) and oral polio vaccine (OPV); and groups 2 and 3 received HBV and OPV only. At nine months of age group 1 received a single standard intramuscular (i.m.) dose of measles vaccine (MV) (Edmonston Zagreb strain, Serum Institute of India Ltd., Pune, India); group 2 received MV and i.m. DTP (Serum Institute of India Ltd.); and group 3

received DTP alone. Outstanding vaccines were administered at 11 months of age (Supplementary Table 1). Venous blood was collected at nine months immediately before vaccination and 4 weeks later: 4.5mLs into a heparinized tube (7.5 units heparin/mL); 0.5mL into a Paxgene^{TM} tube (Qiagen, Crawley, UK).

Whole blood cultures

Heparinized whole blood was cultured in 100µL aliquots in 96-well U-bottom plates with tetanus toxoid (TT) (10µg/ml, Sanofi Pasteur, France); purified protein derivative (PPD) (10µg/mL, Statens Serum Institute, Denmark); a measles peptide-pool of 122 15mer peptides overlapping by 10 amino acids spanning measles protein haemagglutinin (all 1µg/mL final concentration, Sigma-Genosys, UK); heat-killed listeria monocytogenes (HKLM) (10^9 cells/mL) (TLR2 agonist); *E. coli K12* lipopolysaccharide protein S (LPS) (1µg/mL) (TLR4 agonist); flagellin (10µg/mL) (TLR5 agonist); and CLO-75 (10µg/mL) (TLR7/8 agonist) (all from InvivoGen, San Diego, USA). Anti-CD3 (αCD3) (5µg/mL, Becton-Dickinson (BD), USA) and anti-CD28 (αCD28) (5µg/mL, eBiosciences, UK) were used as a general T cell stimulus, and medium alone was the negative control. Plates were incubated for 16 hours at 37°C, 5% CO_2, centrifuged at 2,000 rpm for 5 minutes and 50µL supernatant collected and stored at -20°C.

Multiplex cytokine assays for plasma and culture supernatants

The Bio-Plex 200 Suspension Array system (Bio-Rad, Hercules, California, USA) was used to analyze cytokines according to the manufacturer's instructions (Bio-Rad, Belgium). A human 27-plex array (Bio-plex Pro Human Cytokine 27-plex Immunoassay for fibroblast growth factor basic [FGF basic], eotaxin, granulocyte-

colony-stimulating factor [G-CSF], granulocyte macrophage-CSF [GM-CSF], interferon-gamma [IFN-γ], interleukin-1beta [IL-1β], IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, interferon-gamma-inducible protein-10 [IP-10], melanocortin receptor-1 [MCR-1], macrophage inflammatory protein-1 alpha [MIP-1α], MIP-1β, platelet derived growth factor-BB [PDGF-BB], regulated on activation, normal T cell expressed and secreted [RANTES], tumour necrosis factor [TNF], vascular endothelial growth factor [VEGF]) or a customized 10-plex array (IL-1β, IL-4, IFN-γ, IL-10, IL-12(p70), eotaxin, GM-CSF, PDGF-BB, TNF, VEGF) was used to analyse cytokines in plasma samples (n=214 infants), and cytokines in cultures with vaccine related (TT, Measles Pool) and non-related (PPD, αCD3/αCD28) antigens (n=192 infants). For TLR agonist stimulations, a 5-plex array (IL-1β, IL-6, IL-10, IL-12(p70), TNF) was used (n=122 infants). Assays were not performed for all infants due to insufficient blood volume, clotted or contaminated blood samples. Medium background values were subtracted from antigen-stimulated values to establish net cytokine production. Cytokine ratios were analysed without background correction.

MV and DTP vaccine antibodies

A multiplex microsphere based fluorescent immunoassay for diphtheria toxoid (Dtx), tetanus toxoid (Ttx) and pertussis toxoid (Ptx) IgG antibodies was performed at the National Institute of Public Health and the Environment (RIVM), Netherlands using published protocols [14] (n=284 infants). The measles IgG haemagglutination inhibition assay (HAI) was performed using monkey red blood cells as previously described [15] (n=283 infants).

Statistical analysis of antibody and cytokine data

Cytokine and antibody variables were analysed by fitting a linear mixed model using restricted maximum likelihood. Bleed, group and sex, and their interactions were fitted as fixed effects and infant, and sample within infant, were fitted as random effects. The F-test was used to test for 2-way group-by-bleed and 3-way sex-by-group-by-bleed interactions. Comparisons between treatment groups, and sexes within treatment groups, at each time point, were based on t-tests utilizing the predicted means and standard errors of differences recovered from the fitted mixed model. Comparisons were conducted at 5% significance with no adjustments for multiple comparisons. Cytokine and antibody data required logarithmic transformation prior to analysis. Data were analysed using GenStat 17 (VSN International), R 3.1.2 (www.r-project.org) and Stata version 12.1 (StataCorp LP, USA).

Whole blood RNA microarray analysis

RNA was extracted from Paxgene™ tubes using PAXgene™ blood RNA extraction kits (Qiagen, Crawley, UK) according to manufacturer's instructions. 100ng RNA was used to generate labeled cRNA using the Ambion Illumina TotalPrep-96 RNA Amplification Kit. cRNA was hybridized to Human HT-12 V3 microarrays (Illumina) comprising 47,293 features. 348 RNA samples from 183 infants (165 infants with paired samples) were deemed of sufficient quality for analysis using the array Quality Metrics package in Bioconductor [16]. Raw data were transformed using a variance stabilizing transformation (VST) prior to normalization across all arrays using the robust spline normalization (RSN) method. Eighteen manually chosen comparisons for differentially expressed genes were undertaken using an empirical Bayesian

approach (post- compared to pre- for each vaccine group, for all donors and by sex, for unpaired and paired data). The Benjamini-Hochberg p value adjustment for multiple testing was implemented in limma software package for R, to control for false discovery rate. The resulting gene list was fully annotated and sorted in order of decreasing significance. Functional enrichment analysis was performed using KEGG pathway (www.genome.jp/kegg/) membership (raw $p<0.01$, enrichment $p<0.05$), and Gene Ontology (GO) terms (www.geneontology.org) (raw $p<0.01$, enrichment $p<0.001$). Networks were laid out for all relationships exhibiting a Pearson correlation greater than 0.75. MCL clustering of nodes was undertaken (expansion value 1.7), and functional analysis of each cluster performed using public and proprietary databases. Expression networks were constructed employing the Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com).

RESULTS

Cohort characteristics

302 children were randomized at four months of age into vaccine groups (Fig. 1). Ethnic mix was comparable in the 3 vaccine groups (Table 1). Weight-for-age z scores, haemoglobin and platelet counts were comparable in all groups and sexes (Table 1). MV+DTP males had significantly higher total white cell counts and significantly lower lymphocyte counts after vaccination compared to other groups, although 3-way interactions were not significant. Local HIV seroprevalence [17] and helminth infection rates were low [18] and unlikely to be significant confounders.

Vaccine IgG levels

The expected induction of IgG antibodies to measles occurred in the MV and MV+DTP groups and not the DTP group (Fig. 2A and B). Similarly, diphtheria, tetanus and pertussis toxoid responses were boosted in the DTP and MV+DTP groups, but not the MV group (Fig. 2C-H). Females had higher Ptx antibody levels than males post-vaccination (p=0.01) (Figs. 2G and H), but there were no other sex differences.

Plasma cytokine profiles post-vaccination

Plasma cytokine group differences were found for TNF:IL-10, IFN-γ:IL-10, IL-7, PDGF and IP-10 (Fig. 3). The DTP group had significantly lower IL-7 than the MV group (p=0.029) (Fig. 4A). The DTP group had lower TNF:IL-10 than the other vaccine groups whether for all donors combined, males or females (Fig. 4B). IFN-γ:IL-10 ratios were also lower in DTP males and females, with high levels in MV+DTP females (Fig. 4C). MV males had higher TNF:IL-10 and IFN-γ:IL-10 (p=0.039) than MV females (p=0.011). DTP females had lower IP-10 than DTP males (p=0.037) and MV females (p=0.021) (Fig. 4D). PDGF levels were generally higher in the DTP vaccinated group, and MV females (Fig. 4E).

Toll-like receptor agonist responses

Cytokine responses were tested to TLR2, 4, 5 and 7/8 *in vitro* stimulation, but only TLR4 (LPS) responses changed following vaccination. The DTP group had lower post-vaccination TNF production following LPS stimulation than the MV group (p=0.049) (Figs. 5A). The MV males but not females had increased TNF (p=0.003) and increased TNF:IL-10 (p=0.008) in LPS cultures post- compared to pre-vaccination (Fig. 5B and 5C). In addition, LPS stimulated TNF:IL-10 was

significantly higher in MV males compared to both DTP and MV+DTP males at 10 months (p=0.013 and 0.021, respectively) (Fig. 5C), but this was not observed in females.

Vaccine-specific cellular responses

Priming of measles-specific cellular responses occurred in the MV and MV+DTP but not DTP groups, with upregulation of IFN-γ:IL-10 and IFN-γ:IL-4 to the measles peptide pool post-vaccination (Fig. 6A). TT reactivity was low and not boosted in the DTP vaccinated infants for any of the cytokines tested (*not shown*). There was no evidence of any sex interaction in measles or tetanus specific *in vitro* reactivity.

Cytokine responses to T cell stimulation

Broad T cell reactivity was assessed by αCD3/αCD28 stimulation. Levels of αCD3/αCD28 stimulated eotaxin declined significantly in the MV+DTP group post-vaccination (p<0.001); and post-vaccination levels of eotaxin were lower in this group than the MV (p=0.001) and the DTP groups (p=0.009) (Fig. 6B).

DTP vaccinated females had significantly lower IL-12(p70) levels than males post-vaccination (p=0.022) but not pre-vaccination (p=0.626) (Fig. 6C). Anti-CD3/αCD28 stimulated IFN-γ:IL-4 declined significantly in the DTP group post-vaccination (p=0.029), and post-vaccination levels were lower in the DTP group compared to the MV (p=0.008) and MV+DTP groups (p=0.005) (Fig. 6D). The MV+DTP males had significantly increased αCD3/αCD28 stimulated IFN-γ:IL-4 post-vaccination (p=0.042) (Fig. 6E); and DTP vaccinated females had significantly lower IFN-γ:IL-4 than males at 10 months (p=0.002) but not baseline (p=0.451) (Fig. 6F).

Reactivity to the unrelated antigen PPD

Significant sex-by-bleed-by-group interactions were found for TNF (p=0.001), eotaxin (p=0.004) and IL-10 (p=0.032) following PPD stimulation. For all 3 cytokines the PPD responses were equivalent in males and females at baseline, but increased significantly in MV+DTP females from pre to post-vaccination (p=0.048, 0.02 and 0.043, respectively) (Figs. 7A, C, E), while decreasing in MV+DTP males (p=0.002, 0.024 and 0.016, respectively) (Figs. 7B, D, F). MV males had significantly increased TNF following PPD stimulation post compared to pre-vaccination (p=0.019) (Fig. 7B) whereas females did not (Fig. 7A).

Differential whole human genome RNA expression profiles

Ex-vivo gene expression profiles were analysed to gain further insights into mechanisms of heterologous effects of MV and DTP. While a series of significantly differentially expressed genes were identified (Fig. 8A), after multiple test adjustment only 2 showed statistically significant genes (MV females paired and unpaired - 1 probe of unknown function). Therefore, a less stringent explorative approach accepting a high rate of false discovery was taken. Genes were filtered by fold-change expression post-vaccination compared to pre-vaccination for each group for paired and unpaired data for all donors and males and females separately identifying 70 differentially expressed probes with ≥1.5 fold (up- or down-regulated) in one or more groups (Supplementary Table 2). There was no differential expression ≥1.5 fold unless vaccine treatment groups were separated by sex. Heat maps of the 70 differentially expressed probes were generated, and clustered by genes and conditions using Pearson's dissimilarity (Fig. 8B).

There were 29 probes ≥1.5 fold down-regulated in DTP vaccinated females, the majority being type 1 interferon stimulated genes involved in pattern recognition of pathogens and interferon signalling (Table 2). Ingenuity Pathway Analysis suggested down-regulated interferon signalling and dendritic cell (DC) maturation pathways (Fig. 9A), and the gene ontology terms implicated were recognition of viruses and bacteria. By contrast, DTP vaccinated males had 18 probes ≥1.5 fold up-regulated (Table 2). One major network was produced consisting of up-regulated functions associated with developmental pathways, RNA transcription complex and post-transcriptional modification pathways (Fig. 9B). The top canonical pathway functions were up-regulated assembly of RNA polymerase II complex, T-cell receptor signalling and protein kinase A signalling; the major associated functions being genes involved in developmental and repair functions (Table 2).

While DTP vaccinated males had exclusively up-regulated genes, males vaccinated with MV+DTP had only down-regulated genes (Table 2, Fig. 9C). The top canonical pathway associated functions were for down-regulated amino acid metabolism and signalling processes. There were insufficient differentially expressed genes among MV males and females and MV+DTP females to perform pathway analysis.

DISCUSSION

This is the first longitudinal randomized trial designed specifically to study for heterologous effects of vaccination with MV, DTP or both together. Lower post-vaccination pro-inflammatory responses to TLR4 stimulation in DTP vaccinated infants implies impaired innate immunity to gram-negative bacteria, whereas responses

to TLR2, TLR5 and TLR7/8 were unaffected. BCG vaccination enhances TLR4 reactivity via epigenetic upregulation of NOD2 activity on macrophages, which in turn increases pro-inflammatory responses to unrelated pathogens [19]. Therefore a plausible mechanism for DTP-induced TLR4 inhibitory effects is via epigenetic modulation. The tentative down-regulation of predominantly type 1 interferon genes in DTP vaccinated females but not males further supports this theory, since the STAT1 pathway, which is strongly dependent on type 1 interferons, is a pivotal mechanism for 'trained immunity' leading to altered innate memory [20].

DTP vaccinated females also had suppressed T cell reactivity, as evidenced by decreased αCD3/αCD28 stimulated type 1 cytokines following vaccination. Vaccines may influence T cell responses to unrelated pathogens via induction of cross-reactive T cells that can either be protective or harmful [21]; but suppression of reactivity to anti-CD3/anti-CD28 stimulation suggests a more generalized suppression of T cell immunity. Lower plasma IL-7 in DTP vaccinated infants, a haematopoietic growth factor that plays an important role in T and B cell development and survival [22], could further cause impaired cellular immunity.

These broad immunosuppressive effects of DTP vaccination, particularly in females, could account for increased susceptibility to infections and all-cause mortality following DTP [9, 13]. That these effects were not significant when MV was given with DTP suggests co-administration of the live measles vaccine modifies the immunosuppressive effects of DTP.

Measles vaccination provides beneficial heterologous effects, particularly in females, via unknown mechanisms [6, 8]. In our study, the major MV effect was in males who had more pro-inflammatory plasma and enhanced TLR4 reactivity following vaccination. Therefore modified TLR4 reactivity is major candidate mechanism for heterologous effects of both MV and DTP. Plasma IP-10 was higher in MV females, a Th1 biasing chemokine with a role in effector T cell trafficking [24], but also known to predict severity of a number of inflammatory diseases [25].

Reactivity to the mycobacterial antigen PPD can be due to BCG vaccination and priming by environmental mycobacteria [26]. Enhanced PPD responses in MV+DTP females, and down-regulated in MV+DTP males, suggest that when the vaccines are given together, females immunity becomes more reactive, while males become less so. In keeping with this was down-regulation of several cellular proliferation and growth genes in MV+DTP vaccinated males but not females. MV+DTP males also had higher WBC and lower lymphocytes than other groups post-vaccination, suggesting a bone marrow effect. Negative effects in males concurs with epidemiological data suggesting males may be more susceptible than females to adverse heterologous effects following administration of MV with DTP [10, 11].

Our results highlight the importance of analysing for sex interactions. Males and females are immunologically skewed in opposite directions due to sex hormones, most evident during puberty, but also in infancy [27, 28]. The X-chromosome contains numerous immune response genes and micro-RNAs contributing to immunological sex differences [29, 30].

Several limitations should be noted. Samples were taken at a single time point post-vaccination, and immune dynamics may differ in the sexes. Plasma was only analysed at 10 months but not baseline. The MV group received an additional DTP at four months of age compared to the other two groups. Differentially expressed genes did not reach statistical significance after correction for multiple testing and a less stringent analysis approach was taken.

Our data provide further evidence that vaccines educate both innate and adaptive immunity in previously unsuspected ways [2, 21]. We have identified plausible immunological effects at the protein level underscoring the heterologous effects of MV and DTP vaccines and their sex-differential nature, and differentially expressed genes that might be explored as targets of these vaccine effects. The epigenetic effects of DTP and MV on TLR4 signalling should be explored to investigate for negative and positive innate 'immune training' effects. It will also be crucial to investigate the mechanism of suppressed T cell signalling in DTP vaccinated females. The effects of hormones and X-linked immune response genes could also be explored. The public health implications of understanding these vaccine effects are enormous since they could be exploited in future vaccine design. Furthermore, minor changes to vaccine schedules could maximize beneficial and minimize deleterious effects of vaccines [32].

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Potential conflicts of interest

None

References

1. Aaby P, Kollmann TR, Benn CS. Nonspecific effects of neonatal and infant vaccination: public-health, immunological and conceptual challenges. Nat Immunol, 2014; 15(10): 895-899.

2. Benn CS, Netea MG, Selin LK, Aaby P. A small jab - a big effect: nonspecific immunomodulation by vaccines. Trends Immunol, 2013; 34(9): 431-439.

3. Flanagan KL. Vaccines have sex differential non-targeted heterologous effects: a new dawn in vaccine research. Trans R Soc Trop Med Hyg, 2015; 109(1): 1-2.

4. Flanagan KL, van Crevel R, Curtis N, Shann F, Levy O. Optimmunize Network. Heterologous ("nonspecific") and sex-differential effects of vaccines: epidemiology, clinical trials, and emerging immunologic mechanisms. Clin Infect Dis, 2013; 57(2): 283-289.

5. Aaby P, Roth A, Ravn H, Napima BM, Rodrigues A, Lisse IM, *et al.* Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? J Infect Dis, 2011; 204(2): 245-252.

6. Aaby P, Martins CL, Garly ML, Balé C, Anderson A, Rodrigues A, *et al.* Non-specific effects of standard measles vaccine at 4.5 and 9 months of age on childhood mortality: randomised controlled trial. BMJ, 2010; 341: c6495.

7. Mina MJ, Metcalf CJ, de Swart RL, Osterhaus AD, Grenfell BT. Long-term measles-induced immunomodulation increases overall childhood infectious disease mortality. Science, 2015; 348(6235): 694-699.

8. Aaby P, Samb B, Simondon F, Seck AM, Knudsen K, Whittle H. Non-specific beneficial effect of measles immunisation: analysis of mortality studies from developing countries. BMJ, 1995; 311(7003): 481-485.

9. Aaby P, Benn C, Nielsen J, Lisse IM, Rodrigues A, Ravn H. Testing the hypothesis that diphtheria-tetanus-pertussis vaccine has negative non-specific and sex-differential effects on child survival in high-mortality countries. BMJ Open, 2012; 2(3).

10. Aaby P, Jensen H, Walraven G. Age-specific changes in the female-male mortality ratio related to the pattern of vaccinations: an observational study from rural Gambia. Vaccine, 2006; 24(22): 4701-4708.

11. Aaby P, Vessari H, Nielsen J, Maleta K, Benn CS, Jensen H, *et al.* Sex differential effects of routine immunizations and childhood survival in rural Malawi. Pediatr Infect Dis J, 2006; 25(8): 721-727.

12. WHO, Meeting of the Strategic Advisory Group of Experts on immunization, April 2014 – conclusions and recommendations. 2014; 89(21): 221-236.

13. Aaby P, Ravn H, Benn CS. The WHO review of the possible non-specific effects of diphtheria-tetanus-pertussis vaccine. Ped Infect Dis J. 2016; Epub.

14. Van Gageldonk PGM, van Schaijk FG, van der Klis FR, Berbers GAM. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus. J Imm Methods, 2008; 335: 79-89.

15. Whittle HC, Campbell H, Rahman S, Armstrong JR. Antibody persistence in Gambian children after high-dose Edmonston-Zagreb measles vaccine. Lancet, 1990; 336(8722): 1046-1048.

16. Kauffmann A, Gentleman R, Huber W. ArrayQualityMetrics - a bioconductor package for quality assessment of microarray data. Bioinformatics, 2009; 25(3): 415-416.

17. UNICEF. At a glance: Gambia. 2015; Available at: http://www.unicef.org/infobycountry/gambia_statistics.html. Accessed 27 April 2016.

18. Finney OC, Nwakanma D, Conway DJ, Walther M, Riley EM. Homeostatic regulation of T effector to Treg ratios in an area of seasonal malaria transmission. Eur J Immunol, 2009; 39(5): 1288-1300.

19. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, *et al.* Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci USA, 2012; 109(43): 17537-17542.

20. Yoshida K, Maekawa T, Zhu Y, Renard-Guillet C, Chatton B, Inoue K, *et al.* The transcription factor ATF7 mediates lipopolysaccharide-induced epigenetic changes in macrophages involved in innate immunological memory. Nat Immunol, 2015; 16(10): 1034-1043.

21. Gil A, Kenney LL, Mishra R, Watkin LB, Aslan N, Selin LK. Vaccination and heterologous immunity: educating the immune system. Trans R Soc Trop Med Hyg, 2015; 109(1): 62-69.

22. Dooms H. Interleukin-7: Fuel for the autoimmune attack. J Autoimmun, 2013; 45: 40-48.

23. Jensen KJ, Sondergaard M, Andersen A, Sartono E, Martins C, Garly ML, *et al.* A randomized trial of an early measles vaccine at 4(1/2) months of age in

Guinea-Bissau: sex-differential immunological effects. PLoS One, 2014; 9(5): e97536.

24. Khan IA, MacLean JA, Lee FS, Casciotti L, DeHaan E, Schwartzman JD, *et al.* IP-10 is critical for effector T cell trafficking and host survival in Toxoplasma gondii infection. Immunity, 2000; 12(5): 483-494.

25. Liu M, Guo S, Hibbert JM, Jain V, Singh N, Wilson NO, *et al.* CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. Cytokine Growth Factor Rev, 2011; 22(3): 121-130.

26. Burl S, Adetifa UJ, Cox M, Touray E, Ota MO, Marchant A, *et al.* Delaying bacillus Calmette-Guerin vaccination from birth to 4 1/2 months of age reduces postvaccination Th1 and IL-17 responses but leads to comparable mycobacterial responses at 9 months of age. J Immunol, 2010; 185(4): 2620-2628.

27. Flanagan KL, Jensen KJ. Sex differences in outcomes of infections and vaccinations in under five-year-old children. In: Sex and Gender Differences in Infection and Treatments for Infectious Diseases. SL Klein and CW Roberts, Editors. 2015, Springer International, 273-312.

28. Flanagan KL, Klein SL, Skakkebaek NE, Marriott I, Marchant A, Selin LK, *et al.* Sex differences in the vaccine-specific and non-targeted effects of vaccines. Vaccine, 2011; 29(13): 2349-2354.

29. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. Nat Rev Immunol, 2008; 8(9): 737-744.

30. Pinheiro I, Dejager L, Libert C. X-chromosome-located microRNAs in immunity: might they explain male/female differences? The X chromosome-genomic context may affect X-located miRNAs and downstream signalling,

thereby contributing to the enhanced immune response of females. Bioessays, 2011; 33(11): 791-802.

31. Flanagan KL. Sexual dimorphism in biomedical research: a call to analyse by sex. Trans R Soc Trop Med Hyg, 2014; 108(7): 385-387.

32. Shann F. The heterologous (non-specific) effects of vaccines: implications for policy in high-mortality countries. Trans R Soc Trop Med Hyg, 2014; 109(1): 5-8.

Figure Legends

Figure 1 Participant numbers and drop outs

302 infants were randomized at 4 months of age to vaccine groups, 16 of whom dropped out of the study before the first bleed at 9 months of age. Adequate blood samples were obtained from 273 of the 286 vaccinated infants (95.5%) prior to vaccination at 9 months of age, and 254 (93%) at 10 months of age. Numbers tested in each assay in the different vaccine groups are indicated. Blood volumes were often limited and not all assays could be performed on each sample.

Figure 2 Measles and DTP antibody titres before and after vaccination in females and males

Measles (in $\log_2$ HAI titres), diphtheria toxoid (Dtx, IU / mL), tetanus toxoid (Ttx, IU / mL), and pertussis toxoid (Ptx, EU / mL) IgG levels for males (left panels A, C, E, G) and females (right panels B, D, F, H). Mean values are indicated by the horizontal bar. *** indicates $p<0.001$ from the fitted linear mixed model of log transformed mean antibody values for Dtx, Ttx and Ptx and the logit for measles HAI. HAI = haemagglutination inhibition assay. The horizontal dotted line indicates protective Ab titre cutoff, but there is no accepted protective level for Ptx titres (results are shown for n=283 infants for MV Abs and n=284 infants for DTP Abs).

Figure 3 Summary of vaccine group differences in post-vaccination plasma cytokine levels

The figure summarizes where significant differences were found in post-vaccination plasma cytokine levels between vaccine groups for all donors combined (All), males (M) and females (F) showing that for IL-7, TNF:IL-10, IFN-γ:IL-10 and IP-10 the DTP group generally had lower levels than other vaccine groups, whereas for PDGF the DTP group generally had higher levels.

Figure 4 Plasma cytokine levels at 10 months of age

Plasma cytokine levels were measured at 10 months of age, 4 weeks after the study vaccines were administered. No baseline analysis was performed. The figure shows results for IL-7 (A) and TNF:IL-10 ratios (B), IFN-γ:IL-10 ratios (C), IP-10 (D), and PDGF (E). A fitted linear mixed model of log transformed cytokine values was used to determine significant differences by vaccine group and sex. * $p<0.05$, ** $p<0.01$. The height of the bar indicates the mean value, the error bars indicate SEM (results are shown for n=214 infants).

Figure 5 Cytokine responses *in vitro* to TLR agonist stimulation

Infant whole blood was cultured overnight at 9 and 10 months with TLR2, 4, 5 and 7/8 agonists. Only pro-inflammatory responses to LPS (TLR4 agonist) were altered following vaccination: the TNF response to LPS in all donors combined (A); and the TNF (B) and TNF:IL-10 ratios (C) in males only. A fitted linear mixed model of log transformed cytokine values was used to determine significant differences by vaccine group, bleed and sex. * $p<0.05$, ** $p<0.01$. The height of the bar indicates the mean value, the error bars indicate SEM (results are shown for n=122 infants).

Figure 6 Cytokine responses to measles peptides and αCD3/αCD28 stimulation *in vitro*

Infant whole blood was cultured overnight at 9 and 10 months with a measles peptide pool or the general T cell stimulus αCD3/αCD28 and cytokines measured in culture supernatants. Measles-specific responses were stimulated in the MV and MV+DTP groups, but not the DTP alone group (A). A significant group-by-bleed interaction was found for eotaxin responses to αCD3/αCD28 (B); and there were significant sex-by-group-by-bleed interactions in αCD3/αCD28 cultures for IL-12(p70) (C) and IFN-γ:IL-4 ratios (D-F). A fitted linear mixed model of log transformed cytokine values was used to determine significant differences by vaccine group, bleed and sex. * $p<0.05$, ** $p<0.01$, ***$p<0.001$. The height of the bar indicates the mean value, the error bars indicate SEM (results are shown for n=192 infants).

Figure 7 Cytokine responses to PPD stimulation *in vitro*

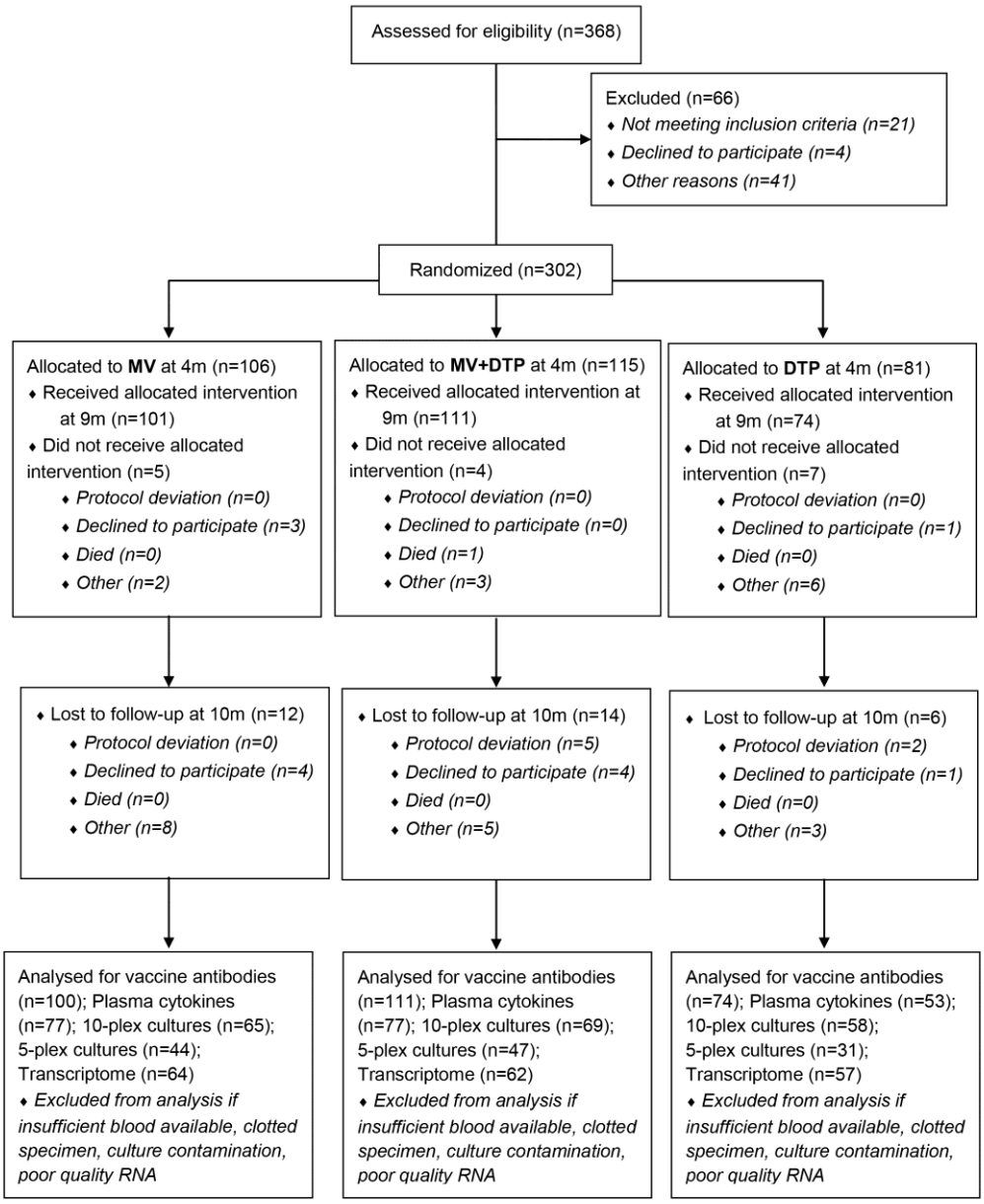
Infant whole blood was cultured overnight at 9 and 10 months with the recall antigen PPD and cytokines measured in culture supernatants. There was a significant group-by-sex-by-bleed interaction for raw TNF (A, B), IL-10 (C, D), and eotaxin (E, F). Female responses are shown in the right hand panels and male responses on the left. Asterisks indicate a significant interaction from the fitted linear mixed model of log transformed cytokine values, * $p<0.05$, ** $p<0.01$. The height of the bar indicates the mean value, the error bars indicate SEM (results are shown for n=192 infants).

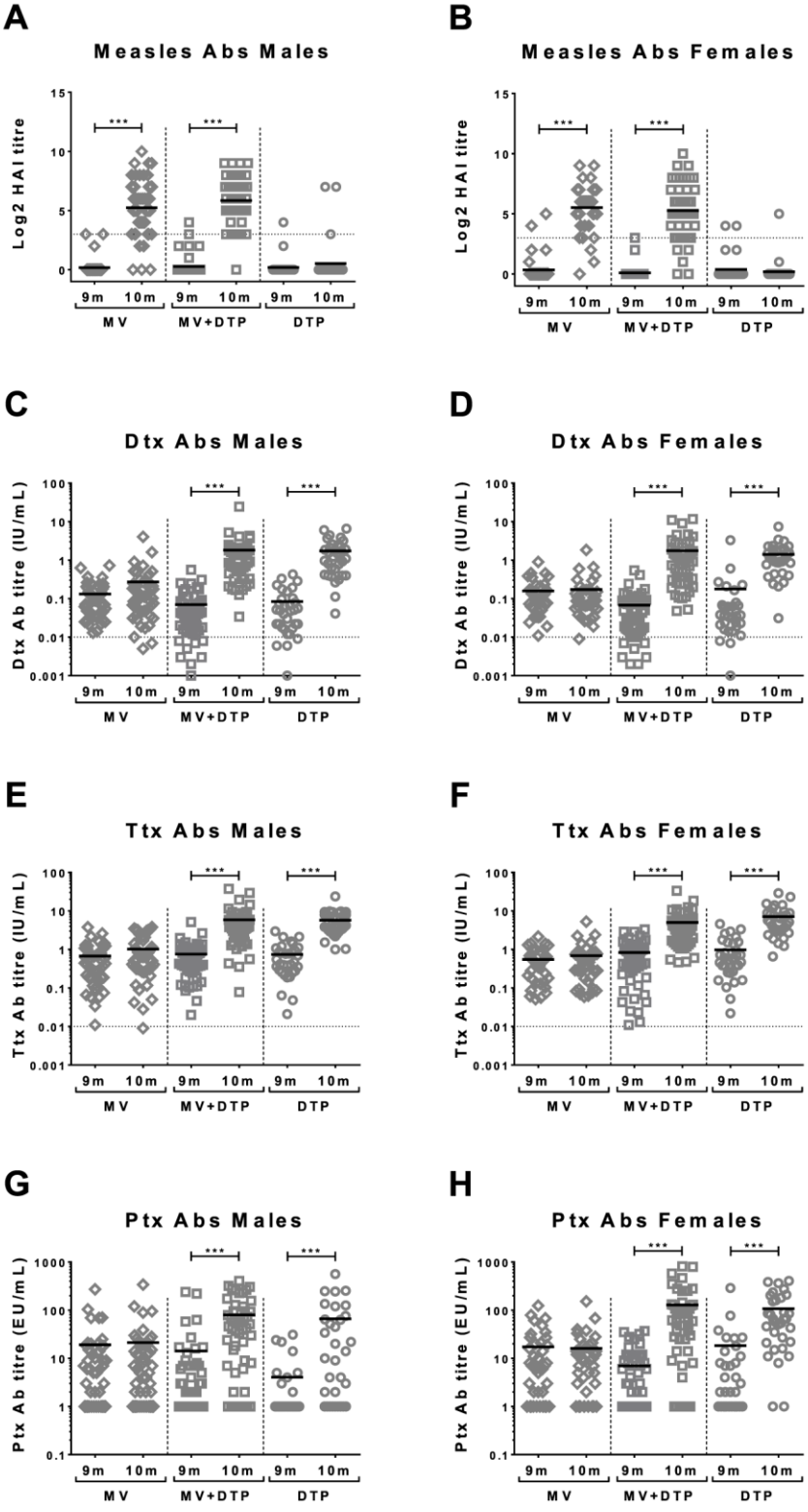
Figure 8 Analysis for differentially expressed probes in the vaccine groups

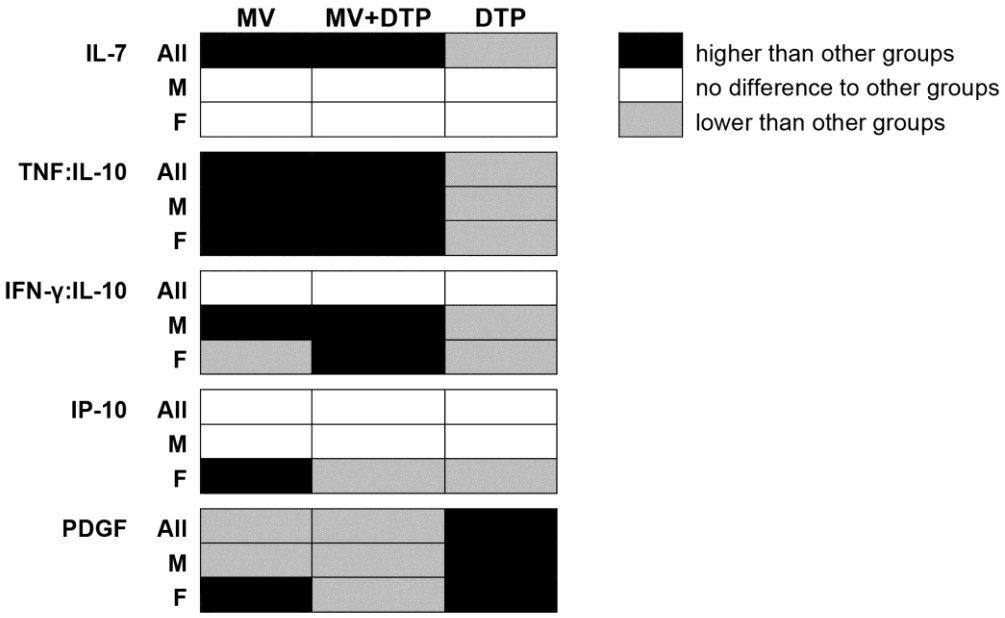
(A) A significance landscape was plotted for the 5,915 non-redundant significant genes (x-axis) by comparisons (y-axis). The number of significantly altered genes in each comparison are shown in parentheses after the comparison description. Comparisons with the highest number of significant changes are shown at the bottom. F = females, M = males, P = paired analysis, where no M or F are shown the analysis is for males and females combined, where no P is shown the analysis is for unpaired data. (B) Hierarchical clustering of the 70 probes with ≥1.5-fold differential expression post-vaccination compared to pre-vaccination organized by vaccine group (left hand y-axis) where purple indicates females and males combined, pink indicates females, and pale blue indicates males. Pairs of data are shown for each group – the upper set representing paired data (P) and the lower set shows unpaired (U) data from all donors. Genes are clustered by Pearson dissimilarity as shown above, values are displayed as log2 fold change in expression. For both panels red = up-regulated, blue = down-regulated, grey = non-significant or no change. Results shown are for n=183 infants.

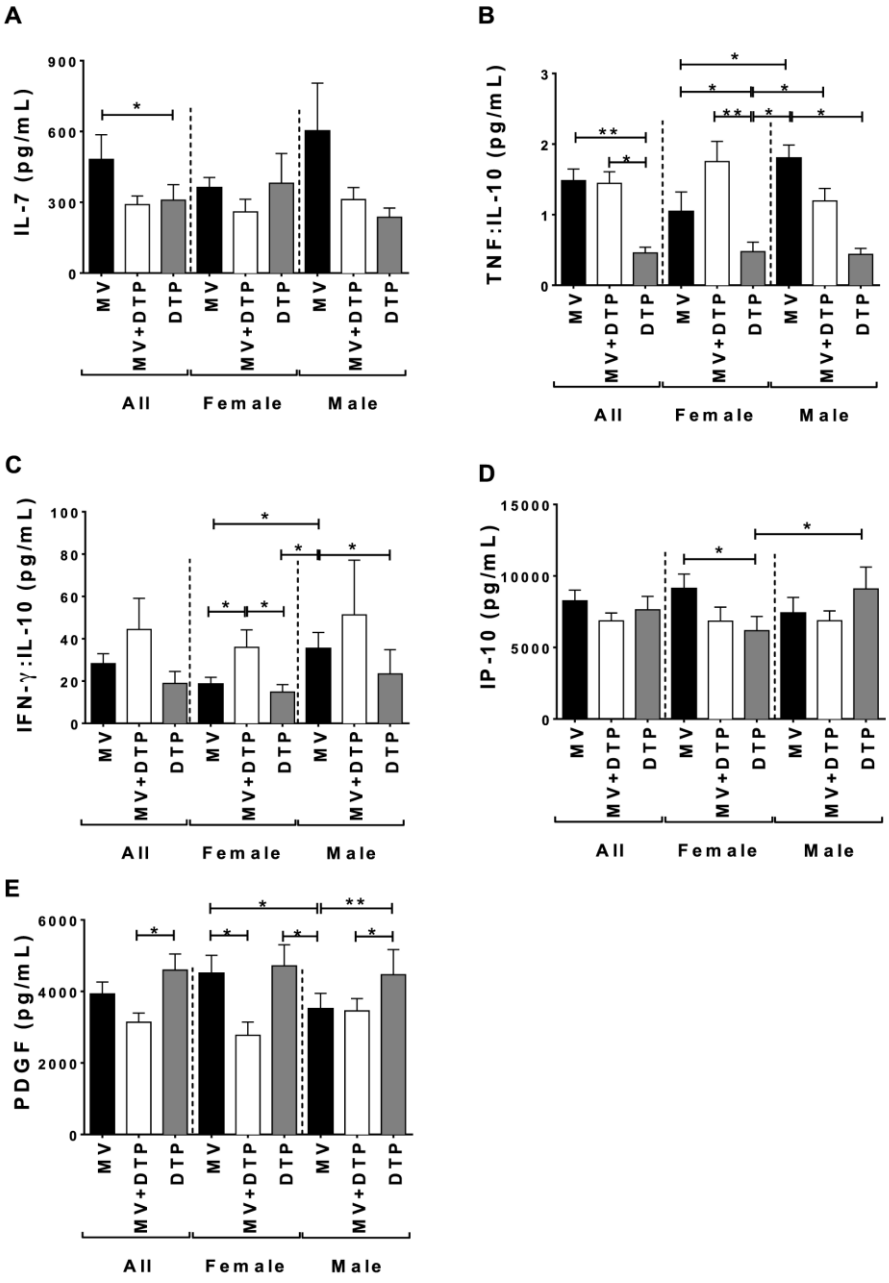
Figure 9 Networks of differentially expressed genes in males and females in the DTP and MV+DTP groups

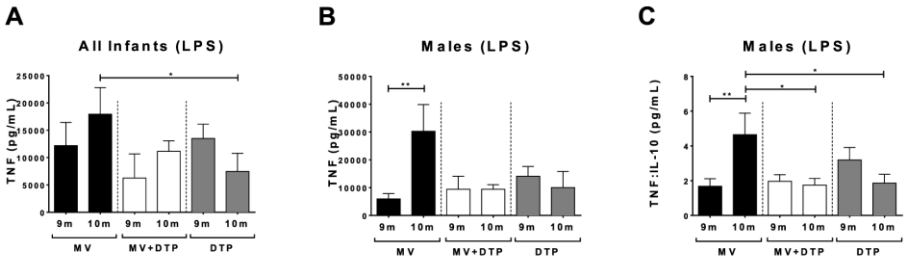
Differentially expressed genes and their associated pathways following DTP vaccination of infant females (A) or males (B), and MV+DTP vaccination of males (C). Networks were generated using Ingenuity Pathway Analysis software. Green nodes indicate down-regulated genes, and red nodes indicate up-regulated genes. There were insufficient differentially expressed genes to generate pathways for the MV males and females, and the MV+DTP females.

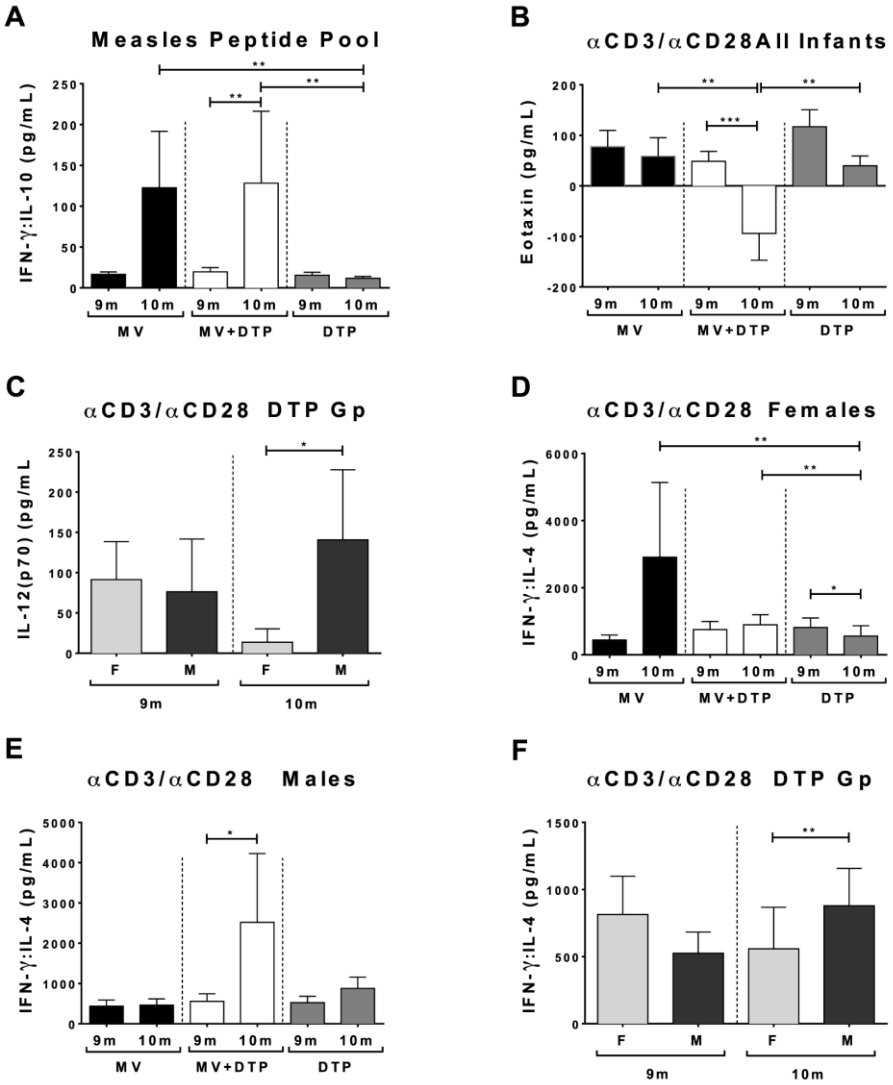


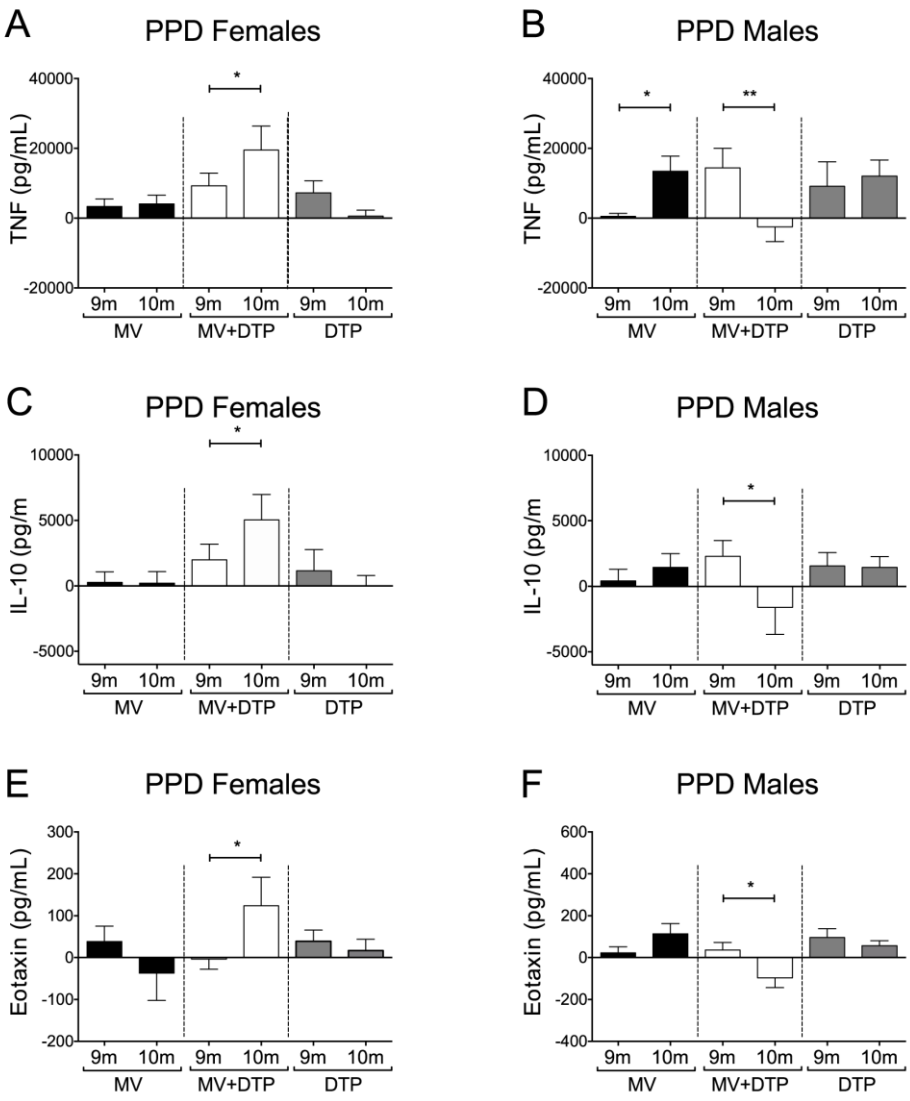












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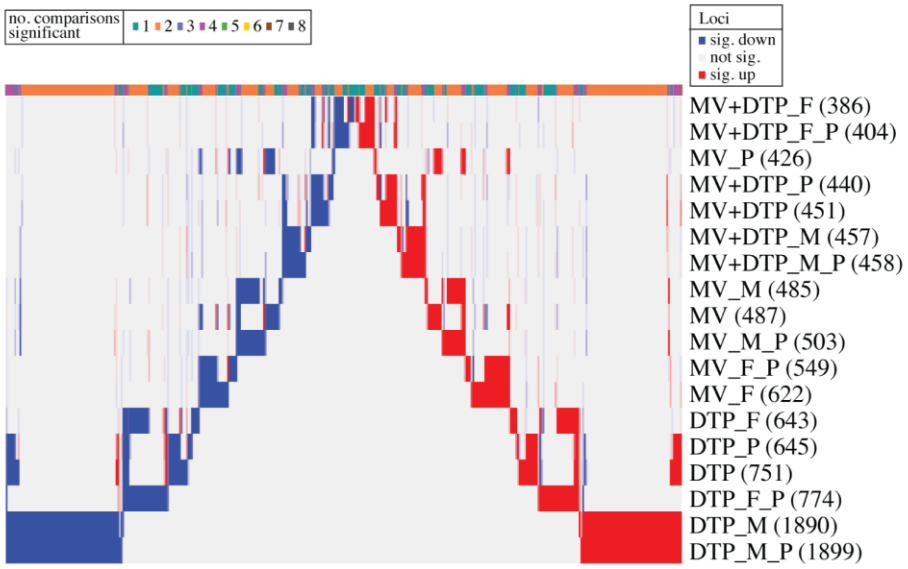

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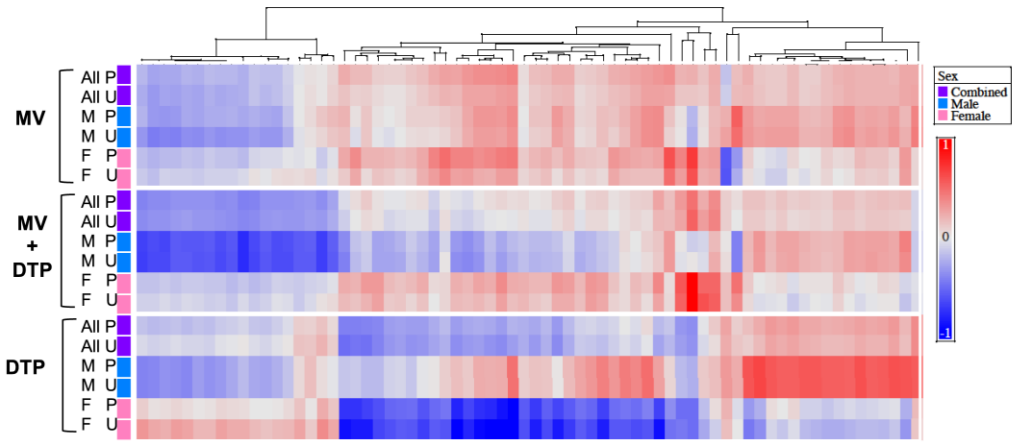

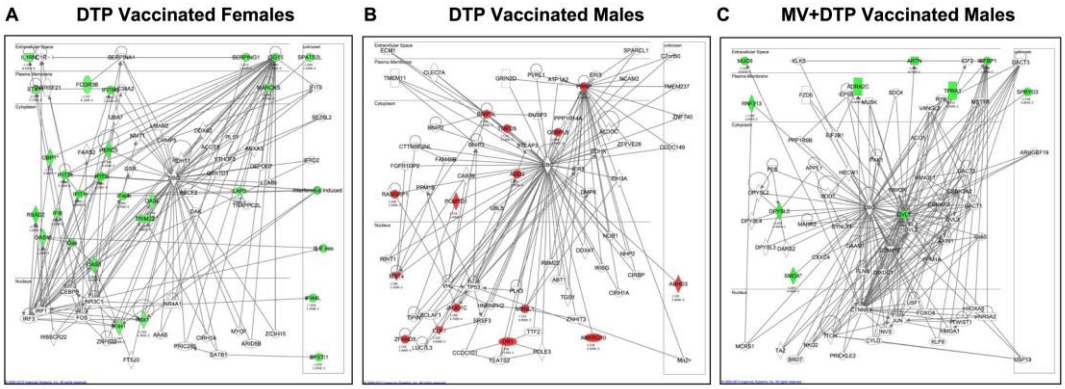

Table 1

Cohort Characteristics and Infant Numbers

	MV Group		MV+DTwP Group		DTwP Group		
	Female	**Male**	**Female**	**Male**	**Female**	**Male**	**Total No.**
No. randomised at 4 months	48	58	56	59	37	44	302
No. infants bled at 9 months	42	55	51	53	32	40	273
No. infants bled at 10 months	36	53	48	49	33	35	254
Weight-for-age z-score at 4 mths	0.22 (-0.56-0.72)	0.20 (-0.46-1.00)	-0.09 (-0.71-0.92)	0.07 (-0.63-0.8)	-0.33 (-0.89-0.28)	0.16 (-0.46-1.02)	
Ethnicity of mother							
Mandinka	38 (79.2%)	42 (72.4%)	47 (83.9%)	41 (69.5%)	27 (75.7%)	29 (65.9%)	
Wolof	1	5	3	3	2	3	
Fula	3	6	3	7	5	5	
Jola	3	2	0	3	1	3	
Other	3	3	3	5	2	4	
Haemoglobin (g/dL)							
9 months	10.2 (9.3-10.8)	10.2 (9.2-11.1)	9.9 (8.7-10.9)	9.8 (8.9-10.9)	10.1 (9.6-10.5)	10.0 (9.4-10.7)	
10 months	10.3 (8.9-10.8)	9.6 (8.6-10.7)	9.5 (8.5-10.2)	9.4 (8.5-10.1)	10.0 (9.0-10.4)	9.7 (8.9-10.4)	
White blood cells (x10^3/μL)							
9 months	8.4 (7.0-10.6)	7.8 (5.3-9.8)	8.8 (7.3-11.4)	8.7 (6.7-12.1)	9.9 (7.1-11.1)	9.2 (6.3-10.2)	
10 months	9.1 (7.2-11.1)	9.3 (7.7-12.3)	9.0 (6.9-10.7)	**11.3 (8.3-13.4)***	8.8 (6.8-11.4)	9.0 (7.0-11.7)	
Lymphocytes (%)							
9 months	63.4 (52.4-68.0)	61.1 (55.8-67.8)	64.8 (59.6-72.1)	62.3 (60.5-66.4)	62.6 (56.3-69.0)	63.4 (50.0-69.1)	
	66.2	63.7	65.5	**59.4**	65.5	66.0	

10 months	(61.7-73.9)	(56.8-67.7)	(59.2-70.5)	**(46.8-66.4)***	(51.6-71.2)	(61.9-68.4)	
Platelets (x10^9/L)							
9 months	165 (104-265)	141 (103-251)	197 (135-272)	183 (121-267)	178 (144-265)	208 (122-263)	
10 months	163 (108-279)	162 (106-234)	153 (123-206)	177 (118-263)	146 (98-252)	196 (126-298)	
No. infants tested in the study assays							
Measles antibodies	44	56	55	54	35	39	283
DTP antibodies	45	55	56	55	35	38	284
Plasma cytokines	32	44	42	43	28	25	214
10-plex culture supernatants	30	35	35	34	28	30	192
5-plex culture supernatants	23	21	22	25	15	16	122
Transcriptome	31	33	30	32	26	31	183

Median and IQ range shown for weight and blood parameters. The donor numbers tested for some assays are higher than the numbers at each bleed because some infants attended for 1 bleed and not the other. The bold text indicates a significant difference in the indicated group compared to other groups.

Table 2 Differentially expressed genes and their functions in the different vaccine groups by sex

Abbrev	Gene Name	Pathway Description	Function	Fold Change
		DTP Vaccinated Females		
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	Interferon signaling	Cell-mediated immune response, Antiviral activity	-2.297
RSAD2	Radical S-adenosyl methionine domain containing 2	Pattern Recognition Receptors in Recognition of Bacteria and Viruses	Cell-mediated immune response, Response to virus	-2.144
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	Interferon Signaling	Molecular Transport, Antiviral defense, Innate immunity	-2
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	Interferon signaling	Antiviral defense, Innate immunity	-1.986
HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5	Protein modification; protein ubiquitination	Post-translational modification	-1.945
IFI44	Interferon-induced protein 44	Activation of IRF by Cytosolic Pattern Recognition Receptors	Response to virus	-1.905
ISG15	ISG15 ubiquitin-like modifier	RIG-I-like receptor signaling pathway; Interferon signaling	Antiviral response	-1.84
OAS3	2'-5'-oligoadenylate synthetase 3	Interferon signaling	Antiviral response	-1.828
IFITM3	Interferon induced transmembrane protein 3	Interferon signaling	Response to virus	-1.828
IFI44L	Interferon-induced protein 44-like	Interferon signaling	Immune response; defense response to virus	-1.828
		DTP Vaccinated males		
ADD3	Adducin 3 (gamma)	Activation of cAMP-Dependent PKA	Hematological System Development and Function; Tissue Morphology	1.625

JMJD1C	Jumonji domain containing 1C	Leukocyte Extravasation Signaling	Hematopoiesis	1.58
MST4	Serine/threonine protein kinase MST4	Apoptotic pathway	Cellular Growth and Proliferation	1.58
ANKRD10	ankyrin repeat domain 10	N/A	N/A	1.558
ZFAND5	Zinc finger, AN1-type domain 5	TNF-alpha/NF-kB Signaling Pathway; FoxO family signaling	N/A	1.558
DR1	Down-regulator of transcription 1, TBP-binding (negative cofactor 2)	Chromatin Regulation / Acetylation	Assembly of RNA Polymerase II Complex	1.548
OSBPL8	Oxysterol binding protein-like 8	N/A	N/A	1.548
RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	Gαs Signaling, T cell receptor signaling pathway	Hematological System Development and Function; Cellular Development	1.548
PRNP	Prion protein	Prion diseases	Cellular Development, Cell Death and Survival	1.537
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	Apoptosis	Cancer; Induces apoptosis	1.526
MV+DTP Vaccinated Males				
SMOX	Spermine oxidase	Amine and polyamine degradation; spermine degradation	Regulation of polyamine intracellular concentration	-1.647
DPYSL5	Dihydropyrimidinase-like 5	Axon guidance	Neural development	-1.636
DVL1	Dishevelled, dsh homolog 1 (Drosophila)	Wnt signaling pathway, Notch signaling pathway, Pathways in cancer	Regulation of cell proliferation, Cancer, Dermatological conditions	-1.613
RNF213	Ring finger protein 213	Protein modification; protein ubiquitination.	Ubiquitin-protein ligase activity	-1.58
SPRYD3	SPRY domain containing 3	N/A	N/A	-1.548
TPRA1	Transmembrane protein, adipocyte asscociated 1	G-protein coupled receptor signaling pathway	Small Molecule Biochemistry	-1.537

MUC6	Mucin 6, oligomeric mucus/gel-forming	N/A	Tissue Development	-1.526
ARTN	Artemin	N/A	Cellular growth and proliferation	-1.516
ADRA2C	Adrenergic, alpha-2C, receptor	Neuroactive ligand-receptor interaction	Nervous system development and function	-1.516
IGFBP1	Insulin-like growth factor binding protein 1	Insulin receptor signaling pathway	Cellular growth and proliferation	-1.505

The top 10 differentially expressed genes are shown for each group